An insight into the proteome of the saliva of the argasid tick *Ornithodoros moubata* reveals important differences in saliva protein composition between the sexes.

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Abstract

Tick saliva contains pharmacologically active molecules that allow these parasites to obtain a blood meal from the host and facilitate host infection by tick-borne pathogens. Recent transcriptomic and proteomic analyses of the salivary glands of several tick species have provided data sets that are invaluable for a better understanding of tick sialomes and tick-host-pathogen relationships. Here we performed a proteomic study of the saliva from the argasid tick *Ornithodoros moubata*. Saliva samples from female and male specimens were analysed separately by LC-MS/MS before and after their equalization to facilitate the identification of the less abundant proteins. We report the array of 193 proteins identified in the saliva of *O. moubata* showing: (i) the broad and complex composition of the saliva of this tick, in good agreement with the complexity of the argasid and ixodid sialomes described previously; (ii) a notable difference in the saliva proteomes of females and males, since only 10 of the proteins identified appeared to be shared by both sexes; and (iii) the presence in the salivary fluid of a wide range of proteins known to be housekeeping/intracellular, which could by secreted in unconventional ways, including exosome secretion.

Keywords: *Ornithodoros moubata*; saliva; protein equalization; proteome; sialome; LC-MS/MS

1. Introduction

Ticks are haematophagous ectoparasites of terrestrial vertebrates of great medical and veterinary importance, mainly because they are vectors of diseases affecting humans, livestock and companion animals. Moreover, tick feeding can cause direct damage to their hosts such as significant blood loss as well as paralysis, toxicosis, irritation and allergy [1]. Tick saliva is known to contain anti-haemostatic, anti-inflammatory and immunomodulatory molecules that modify the physiology of their hosts at the tick bite site, allowing these parasites to obtain a blood meal from the host [2-4]. In addition to their role in feeding and other functions related to ion- and water-handling, tick saliva may potentiate the transmission and establishment of tick-borne pathogens, and therefore immune responses to tick saliva can confer protection against pathogen transmission [4-6]. Accordingly, the identification and characterization of tick salivary proteins may lead to the discovery of novel pharmacological agents and antigen targets for the development of vaccines against ticks and/or tick-borne diseases [4, 7].

Recently, salivary gland transcriptomic and proteomic analyses of several hard and soft ticks have been performed, providing data sets that are invaluable for a better understanding of tick sialomes and the immunobiology at the tick-host-pathogen interface [2, 8, 9]. These sialomes show that the repertoire of tick salivary gland transcripts and proteins is much more broad and complex than anticipated, containing hundreds to thousands of different proteins, many of which are novel, since they have no similarities to other proteins in the data bases. The authors of those studies classified the salivary transcripts and proteins they found as putative secreted or possible housekeeping groups, and then into different groups according to their known or predicted biological function. Most such putative secreted proteins have unknown functions but, if secreted into their hosts, they probably have antihaemostatic, anti-inflammatory, immunomodulatory, or even antiangiogenic or antimicrobial activity [9]. Regarding the probable housekeeping proteins identified, the authors of these studies do not comment further on them but suggest that their sequences may help to identify novel secreted protein families if identified in proteome experiments [2].

The argasid tick *Ornithodoros moubata* is distributed throughout South and East Africa and Madagascar, where it colonizes wild and domestic habitats and feeds mainly on warthogs, but also on domestic swine and humans [10-11]. *O. moubata* transmits important human and animal diseases such as East African tick-borne relapsing fever [12] and African swine fever [13-14], whose control and prevention would largely benefit from the elimination of *O. moubata* from at least synanthropic environments (human dwellings and pigsties).

Previous attempts to develop anti-*O. moubata* vaccines using tick salivary antigens have provided encouraging results [15-16], but an effective vaccine is currently still lacking. It is to be expected that a better knowledge of *O. moubata* salivary proteins engaging at the tick-host-

pathogen interface will result in the identification of novel antigenic targets for new and more effective vaccine developments. To this end, the first approach to the sialome of *O. moubata* consisted of the proteomic analysis of its salivary gland extract (SGE) by 2-D electrophoresis and a 2-D western blot followed by matrix-assisted laser desorption/ionization with tandem mass spectrometry (MALDI-MS/MS) of the most abundant and antigenic spots. This resulted in the identification of only two proteins, moubatin and up to 17 isoforms of TSGP1, showing further that TSGP1 was hyperabundant in SGE [17]. Later, a similar study was performed on equalized SGE, which allowed the identification of some additional low-abundance proteins [18].

In order to expand our knowledge of the actual secreted proteins from the sialome of *O. moubata*, we carried out a proteomic study of the saliva of this tick instead of its SGE. Saliva samples from female and male specimens were analysed separately, since previous evidence obtained by us suggested that their protein repertories could differ between the sexes (unpublished data). In addition, the saliva samples were analysed before and after equalization in order to facilitate the identification of the least abundant proteins. Here we report the array of proteins identified in the saliva of *O. moubata* adults showing: (i) a notable difference in the protein composition of the saliva between the sexes; and (ii) the presence in the salivary fluid of a wide range of proteins previously predicted to be housekeeping/intracellular.

These findings expand our knowledge of soft tick sialomes and may help in the identification of proteins that are secreted to saliva by non-classical ways and might play non-described functions at the tick-host interface, raising new questions about the biology of soft tick saliva and soft tick-host relationships.

2. Material and methods

2.1. Ticks

The *O. moubata* ticks used in this work came from a colony maintained in our laboratory, which was established in the mid 1990s from specimens submitted from the Institute for Animal Health, Pirbright, Surrey, UK. The ticks are fed regularly on rabbits and kept at 28 ºC, 85 % relative humidity and 16 h light / 8 h darkness.

All animal manipulations were done according to the rules from the ethical and animal welfare Committee from the Institution where the experiments were conducted (IRNASA, CSIC), following the corresponding EU rules and regulations.

2.2. Saliva collection

Saliva samples were obtained separately from female and male ticks that had been reared in similar conditions: all of them were the same age and had been fed two times on the

same rabbit hosts, and at the moment of saliva collection they had been starved for four months. For the sake of ease of handling, the ticks were processed in batches of five ticks per batch. Saliva collection was performed as previously described [19-20], with some modifications. First, the ticks were washed three times in warm water and dried on a paper towel. Then, they were held with their dorsum adhered by double sided sticky tape on a glass slide, and 1 µl of a 1 % solution of pilocarpine hydrochloride (Sigma) in phosphate buffered saline pH 7.4 (PBS) was injected into the tick genital pore with a 5 µl Hamilton syringe. Shortly after stimulation, small droplets of clear viscous saliva started to be secreted, which were immediately harvested from the tick mouthparts using a micropipette, and deposited on 200 µl of ice-cooled PBS. Saliva was collected continually from the five ticks in the batch until perceptible emission stopped, some 30–40 minutes after stimulation. Possible contamination from external sources was prevented by the investigators using sterilized material and wearing gloves and head caps. Each batch of saliva was then filtered through a 0.22-µm pore filter (Costar-Corning Inc.), its protein concentration measured in a NanoDrop 2000 spectrophotometer, and stored at -20 ºC until further use. The protein concentration of several successive saliva batches prepared was similar and was found to be, on average, 1.14 µg/µl for females (44 µg of salivary protein per tick), and 0.77 µg/µl for males (31 µg of salivary protein per tick). Reproducibility among saliva batches was assessed by standard SDS-PAGE of some batches selected randomly from each sex and found to be similar within the same sex but different between sexes (Results section; Fig. 1A). To check reproducibility among the different protocols of the induction of salivation, three additional batches of ticks of every sex were induced to salivate by injecting the ticks with 10 µl of a 0.2 % solution of dopamine (Sigma) in PBS [21] into the tick genital pore. The secreted saliva was collected and analysed as described before. The protein concentration of these saliva samples was found to be on average 0.33 µg/µl for females (9.9 µg of salivary protein per tick), and 0.25 µg/µl for males (7.5 µg of salivary protein per tick), and their composition was found to be similar to that of the pilocarpine-induced samples. Accordingly, owing to the higher protein yield of the pilocarpine-based protocol, this protocol was the one applied in ensuing experiments in order to obtain up to 3 mg of saliva protein from each sex. Then, the saliva samples collected were pooled by sex, and an aliquot of each pool was removed and stored at -20ºC (native saliva). The remainder of both pools was subjected to protein equalization.

2.3. Protein equalization of the saliva samples

Before equalization, both saliva pools were lyophilized and re-suspended in water (in 1/10 of the initial volume) in order to concentrate them. Following this, they were dialyzed in PBS using the 3.5 K Slade-A-Lyzer Mini kit (Pierce) for two hours at room temperature (with one buffer change at 60 min). The saliva samples (3 mg of protein/sample) were then equalized using the ProteoMiner small capacity kit (BIO-RAD) following the instructions provided by the

supplier. Both the unbound proteins (unbound) and the proteins retained and later eluted from the column (equalized) were collected and their protein concentrations measured in a NanoDrop 2000 spectrophotometer. Samples of native saliva, equalized saliva, and unbound salivary proteins from both sexes were analysed by standard SDS-PAGE in silver-stained 5–20% gradient gels.

2.4. Trypsin digestion, liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Samples of native and equalized saliva from both female and male *O. moubata* ticks were subjected to proteomic analysis by trypsin digestion in solution followed by LC-MS/MS of the tryptic peptides.

For trypsin digestion in solution, saliva samples containing 10 µg of protein were precipitated overnight at 4 ºC in 10% trichloroacetic acid (TCA). The pellets were washed with 20% acetone at -20 ºC and dissolved in 20 µl of 50 mM NH_4HCO_3 containing 50% trifluoroethanol (TFE). Then, they were reduced with 10 mM dithiothreitol (DTT) for 30 min at 60ºC, and alkylated with 55 mM iodoacetamide for 30 min at room temperature in the dark. Following this, proteins were digested overnight at 37ºC with 2.5 ng/µl of sequencing grade trypsin (Promega) in a total volume of 200 µl of 50 mM NH_4HCO_3 and 5% TFE. The reaction was stopped with 20 µl of 10% trifluoroacetic acid (TFA) and the supernatants were filtered through a 0.22 µm filter and dried by centrifugation in vacuum.

LC-MS/MS was performed as described before [22]. Briefly, the resulting peptides from the above-mentioned digestions were resuspended in 6 µl of 5% acetonitrile, 0.1% TFA and 5 µl of the sample was loaded onto a trap column (PepMap C18, 300 µm×5 mm, LC Packings) and desalted with 0.1% TFA at a flow rate of 30 µl/min for 3 min. The peptides were then loaded onto an analytical column (PepMap C18 3 µ 100 Å, 75 µm× 15 cm, LC Packings) equilibrated in 5% acetonitrile and 0.1% formic acid. Elution was carried out with a linear 5–40% gradient of solvent B (95% acetonitrile, 0.1% formic acid) for 120 min at a flow rate of 300 µl/min. The peptides eluted were analysed with a nanoESI-Q-TOF mass spectrometer (QSTAR-XL, AB Sciex) in an information-dependent acquisition mode (IDA), in which a 1-s TOF MS scan from 400–1800 m/z was performed, followed by 3-s product ion scans from 65–1800 m/z on the three most intense doubly- or triply-charged ions.

2.5. Database searching and protein identification

Database searching and protein identification were performed as described before [22]. Protein Pilot v2.0 (Applied Biosystems) was used to search FASTA protein databases and Mascot v2.2 (Matrix Science) was used to mine EST databases.

Protein Pilot default parameters were used to generate peak lists directly from QSTAR wiff files of saliva samples. The Paragon algorithm of Protein Pilot was used to search the

NCBInr protein database with the following parameters: trypsin specificity, cys-alkylation and restricted taxonomy to metazoans. To avoid using the same spectral evidence in more than one protein, the proteins identified were grouped based on MS/MS spectra by the Protein-Pilot Progroup algorithm. Thus, proteins sharing MS/MS spectra were grouped, regardless of the peptide sequence assigned. The protein within each group able to explain more spectral data with confidence was designated as the primary protein of the group. Only the proteins of the group for which there was individual evidence (unique peptides with enough confidence) were also listed. Only primary proteins are shown in the results. A Protein Pilot unused score above 1.3, which is equivalent to a protein confidence threshold greater than 95%, was considered significant. Under these conditions the calculated false discovery rate (FDR) using a reverse decoy database was under 1%.

For Mascot searches, the peak lists were generated directly from QSTAR wiff files by Mascot Daemon v 2.2.2 (Matrix Science) with Sciex Analyst import filter options, using the default parameters. The EST_acari (2378274 sequences; 545902716 residues) database was searched using the following parameters: tryptic specificity, allowing two missed cleavages and a tolerance on the mass measurement of 80 ppm in MS mode and 0.5 Da for MS/MS ions. The carbamidomethylation of Cys was set as a fixed modification, and Met oxidation and Asn/Gln deamidation were set as variable modifications. The significant threshold was set to 0.02 to keep the FDR, calculated by Mascot using a decoy database, below 5%. Only proteins for which there were two or more red bold matches were selected and shown in the results.

The relative abundance of a protein in the sample was quantified using the protein abundance index (PAI), which is defined as the number of observed peptides in the experiment divided by the number of observable tryptic peptides for each protein within a given mass range of the mass spectrometer employed [23]. PAI was modified exponentially to give emPAI, the exponential form of PAI minus one, which is directly proportional to the protein content in a sample [24, 25]. The protein content in a particular sample was calculated as a percentage by dividing the emPAI value of a protein by the sum of all emPAI values in that sample multiplied by 100.

2.6. Bioinformatics, functional annotation and classification of the proteins

For functional annotation of the proteins, blast tools were used to compare the protein sequences to the NR protein database of the NCBI (http://www.ncbi.nlm.nih.gov/), the GO (http://amigo.geneontology.org) and the UniProtKB databases (http://www.uniprot.org), and to search for conserved protein domains in the Pfam, SMART, Kog, and conserved domains (CDD) databases (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). In addition, the following predictions were also performed: presence of signal peptide using SignalP 3.0 [26] at http://www.cbs.dtu.dk/services/SignalP; non-classical protein secretion using SecretomeP 2.0

[27] at http://www.cbs.dtu.dk/services/SecretomeP/; presence of transmembrane helices using the TMHMM Server v. 2.0 at http://www.cbs.dtu.dk/services/TMHMM-2.0; and presence of glycosyl-phosphatidyl anchor sites using the big-PI Predictor [28] at http://mendel.imp.ac.at/sat/gpi/gpi_server.html. Functional annotation of the proteins identified was based on all the above comparisons and on the previously published tick sialomes [2, 8, 9, 29, 30], which served as models for the classification of proteins as either secretory or intracellular/housekeeping, with further subdivisions based on function and/or protein families. Identified proteins with unknown ontology were annotated as "unknown function".

2.7. Protein identification using 1-D gel electrophoresis and LC-MS/MS.

Native saliva samples of both sexes were electrophoresed in 5-20% gradient polyacrylamide gels and stained with Sypro Ruby (SIGMA) according to the manufacturer's instructions. Sypro Ruby-stained gels were digitalized with the ChemiDoc system (Bio-Rad) and gel images were analyzed with Image Lab software (Bio-Rad), including the assessment of the relative abundance of the bands detected. After that, the stained gel bands (approximately 13 in females and 16 in males; see Results section; Fig. 4), were excised and submitted to the Proteomic Service of the Universidad de Valencia (Spain).

There, they were subjected to trypsin digestion and LC-MS/MS analysis for protein identification. Briefly, gel slices were conditioned with 50% acetonitrile, dried and digested with trypsin (20 ng/µl in 25 mM NH_4HCO_3) overnight at 37ºC. The reactions were stopped with 10% trifluoroacetic acid (TFA) to a final concentration of 0.1%, and the supernatants were filtered through a 0.22 µm filter and dried by centrifugation in vacuum. The resulting peptides were resuspended in 6 µl of 5% acetonitrile, 0.1% TFA and 5 µl of the sample was loaded onto a trap column (NanoLC Column, 3µ C18-CL, 75 µm×15 cm, Eksigen) and desalted with 0.1% TFA at a flow rate of 2 µl/min for 10 min. The peptides were then loaded onto an analytical column (NanoLC Column, 3µ C18-CL, 75 µm×25 cm, Eksigen) for 10 minutes at 3 µl/min. Elution was carried out with a linear 5–40% gradient of solvent B (95% acetonitrile, 0.1% formic acid) for 30 min at 300 nl/min, and the peptides eluted were analysed with a nanoESI-Q-TOF mass spectrometer (5600 TripleTOF, AB Sciex) in an information-dependent acquisition mode (IDA). The 50 most intense precursors in each TOF MS scan were selected for fragmentation. Combined MS and MS/MS spectra were searched against the NCBInr_metazoa and EST_acari databases using the Mascot v 2.3.02 algorithm with the following parameters: tryptic specificity, allowing two missed cleavages and a tolerance on the mass measurement of 50 ppm in MS mode and 0.5 Da for MS/MS ions. The carbamidomethylation of Cys was set as a fixed modification, and Met oxidation and Asn/Gln deamidation were set as variable modifications. The significant threshold was set to 0.05 and only proteins for which there were one or more red bold matches were selected and shown in the results.

2.8. Comparative immune-detection of the actin and enolase proteins in the saliva from female and male O. moubata ticks by Western blot

For the immune-detection of the actin and enolase proteins in *O. moubata* female and male saliva, the corresponding polyclonal sera against the *O. moubata* recombinant actin and enolase proteins were obtained from rabbits [31].

Briefly, the whole actin and enolase cDNA coding sequences were amplified by reverse transcription polymerase chain reaction (RT-PCR) using two sets of specific primers designed *ad hoc* on the respective nucleotide sequences retrieved from the GenBank: Actin (GenBank ID: AB208021.1), Enolase (GenBank ID: GU594041.1). The PCR products were purified, cloned in the pSC-A sequencing vector (Stratagene) and then subcloned in the pQE-30 expression vector (Qiagen). Recombinant protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) and the recombinant proteins were purified using nickel-affinity columns. All the above-mentioned experiments were performed following standard procedures [11].

The purified recombinant proteins were inoculated emulsified with Freund's adjuvant into New Zealand white rabbits, as described elsewhere [32]. Each animal received three doses of 200 µg/dose of the corresponding recombinant protein administered at two inoculation points at 15-day intervals. Rabbits were bled immediately before the administration of the first dose and at seven days after the third dose. Blood samples were allowed to clot and sera were removed and stored at -80ºC. IgG antibody levels against the recombinant proteins were titrated by ELISA in two-fold dilution serum series according to standard procedures [16], and were found to be higher than 1/12,800.

For western blots, 15 µg samples of female and male native saliva and 1 µg samples of the corresponding recombinant proteins were resolved by SDS-PAGE in 5–20% gradient gels and electrotransferred to nitrocellulose membranes at 400 mA for 90 min. Blots were blocked with 1% BSA in PBS and then rinsed with PBS containing 0.05% Tween 20. Following this, the sheets were incubated with either the corresponding anti-recombinant rabbit polyclonal serum or with a pool of the sera from two pigs sensitized by natural infestations (both at 1/100 dilution). These latter sera were obtained in earlier studies and had been preserved at -80 ºC [33]. After three new washes, the blots were incubated with horseradish peroxidase (HRP)-labelled anti-rabbit IgG or HRP-anti-pig IgG (both from Sigma) at 1/2,000 dilution and washed again three times. Incubations were performed at 37ºC for 1 h, and the washes were carried out at room temperature over 10 min for each wash. Finally, the recognized bands were developed with 4-Cl-1-naphthol. Pre-immune rabbit and pig sera were reacted in parallel as negative controls (not shown).

3. Results

3.1. SDS-PAGE of native and equalized saliva from female and male O. moubata ticks

The protein band patterns of several batches of native saliva from both sexes were very similar within each sex and noticeably different between the sexes (Fig. 1A). Some 20 bands were found in female saliva, with molecular weights ranging between 150 and 15 kDa. The bands from 25 to 20 kDa were by far the most intense. By contrast, in the saliva from males up to 36 bands were observed, with molecular weights ranging from more than 250 to 15 kDa. Among them, the most intense bands were seen at around 100, 37 and 25-20 kDa.

Equalization increased the number and intensity of perceptible bands in the saliva of both sexes, resulting in more complex band patterns, which at first sight looked quite similar to each other (Fig. 1B). However, closer inspection revealed that there were still significant differences between them.

3.2. Number of proteins identified by LC-MS/MS in native and equalized saliva samples

Overall, for the four types of saliva samples analysed Mascot searching of the EST_acari database retrieved 278 protein hits, 187 of which could be identified. The remaining 91 protein hits (33%) could not be identified because they did not match any known sequence in the blast analysis. Protein pilot searching of the protein NCBInr database retrieved 154 supplementary identifications. Thus, global database searching resulted in 341 protein identifications. After removal of the redundant identifications in each sample, a final count of 203 identified proteins was obtained.

For each saliva sample, the number of protein hits, the number of identified proteins, and the number of non-redundant identifications are indicated in Table 1. The proteins identified simultaneously in native and equalized saliva of the same sex (three in females, 11 in males) were counted only once and annotated to the native samples. Thus, the proteins annotated to the corresponding equalized samples were always additional identifications. As a result, we identified 118 proteins in female saliva (69.5% of them only after equalization), and 85 proteins in male saliva (40% of them only after equalization). Accordingly, equalization increased the number of protein identifications by 228% in females and 67% in males.

A total of 193 different proteins were identified in the saliva of *O. moubata* adults, with only 10 proteins present simultaneously in both sexes.

3.3. Description and classification of the proteins identified in the saliva of O. moubata.

The proteins identified were classified as putative secreted or possible intracellular/housekeeping proteins, and were then divided into groups according to their molecular function or family (Tables 2 and 3), in a similar way as was done for the above-

347 referred tick sialomes (section 2.6.). As a result, some 25% of the identified proteins were
348 classified as secreted, while the remaining ones (approximately 75%) were classified as
349 housekeeping/intracellular (Fig. 2). Subsequent analysis with SecretomeP predicted that at least
350 one-third of these possible housekeeping/intracellular proteins could have been secreted
351 unconventionally (Tables 2 and 3). For the remaining two-thirds, the bioinformatics tools used
352 did not predict any secretion method, despite the fact that they all were found in a secreted fluid.
353 Regarding protein function, among the putative secreted proteins, the most numerous in
354 both sexes had unknown ontologies and were annotated as "unknown function". The remaining
355 proteins were distributed in six functional groups; these groups were the same in each sex and
356 contained similar numbers of proteins, enzymes (24%–30%) and the lipocalins (10%–12%)
357 being the most numerous, followed by the immunity-related, glycine-rich, mucins and protease
358 inhibitors (Fig. 2).
359 Among the putative intracellular/housekeeping proteins, the most numerous in both
360 sexes were also the annotated as "unknown function". The others were distributed in 11–12
361 groups, which were the same in both sexes except for the nuclear export machinery group,
362 which was only found in males. The proteins involved in signal transduction (15%), nuclear
363 regulation (14%), protein synthesis (11%) and transporters (11%) were the most numerous in
364 females, while those involved in protein synthesis (16%), metabolism (13%), energy
365 metabolism (9%) and signal transduction (8%) were the most numerous in males. Cytoskeletal
366 proteins were the next most numerous proteins in both female and male saliva (5.4% and 5%,
367 respectively), actin being the only cytoskeletal protein shared by both sexes. The remaining
368 groups, six in females and seven in males, respectively represented 25% and 22% of the
369 putative housekeeping proteins identified (Fig. 2), and contained only one protein shared by
370 both sexes (the RNA-binding protein musashi; Tables 2 and 3).
371 Additionally, 3 proteins from host blood were also found in the *O. moubata* saliva:
372 serum albumin in females, and haemoglobin alpha and beta chains in males, which are classified
373 as "host origin proteins" in Tables 2 and 3, although they are not included in Fig. 2. Since tick
374 saliva is known to contain ingested host proteins, including albumin and immunoglobulins [34,
375 35] the finding of these proteins was not unexpected, although they were not considered further
376 in the current study.
377
378 *3.4. Relative quantification of the proteins identified in the saliva of O. moubata.*
379 The exponentially modified protein abundance index (emPAI) and % emPAI values
380 obtained for some of the proteins identified in the native saliva samples (Tables 2 and 3)
381 provided information about their relative amounts in this secretion [24, 25]. According to these
382 emPAI values, the most abundant proteins in female saliva were by far the lipocalins, which
383 represented the 100% of the emPAIs computed (Table 2, Fig. 3). By contrast, in the male saliva

the most abundant proteins were the group of enzymes involved in energy metabolism, followed by lipocalins, which totalled 55.55% and 30.5%, respectively, of the emPAI values computed (Table 3 and Fig. 3). The proteins in the native saliva samples without an emPAI value and the proteins that were only identified in the equalized samples (with or without emPAI value) were assumed to be low or very low in abundance (Tables 2 and 3).

The % emPAI-based relative quantification paralleled the results of the quantification carried out on Sypro Ruby-stained 1-D polyacrylamide gels (Fig. 4). After the analysis of the relative abundance of the Sypro Ruby-stained bands, the proteins present in these bands were identified by LC-MS/MS analysis (Supplementary table 1), depicted in Fig. 4 and noted down in tables 2 and 3. As can be observed in Fig. 4, bands containing lipocalins represented up to 93.1% of the protein mass in the female saliva, while in male saliva the most abundant bands were those containing the enzymes involved in energy metabolism (more than 51.6%), followed by the bands corresponding to lipocalins (31.5%).

3.5. Detection of the actin and enolase proteins in O. moubata saliva by western blot

This experiment was carried out to confirm, in a different way, the results of proteomics regarding the presence of actin in the saliva of both sexes and the presence of enolase in males only. As shown in Fig. 5A, recombinant actin and the native actin in female and male saliva were all recognized by the anti-recombinant actin hyperimmune rabbit serum. The slightly larger size of the recombinant actin can be attributed to the histidine tag from the pQE-30 expression vector. By contrast, Fig. 5B shows that the anti-*O. moubata* sera obtained from pigs sensitized by natural contact did not recognize either the recombinant actin or the actin band in the saliva lanes, while they did recognize the natural immunogenic proteins. Fig. 5B also shows notable differences in antigenic band patterns between sexes, reinforcing the earlier evidence of their different saliva composition. Thus, these results confirmed the real presence of actin in the saliva of both sexes and suggest that native actin is not immunogenic through natural contact.

Regarding enolase, Fig. 6A shows that the anti-recombinant enolase hyperimmune rabbit serum recognized the recombinant enolase and a band of native enolase in male saliva, but not in female saliva. As was the case for actin, the histidine tag accounted for the slightly larger size of the recombinant enolase *vs.* native enolase. Fig. 6B, shows that the anti-*O. moubata* sera obtained from pigs sensitized by natural contact did not recognize either the recombinant enolase or the native enolase in the male saliva, whereas they recognized the same immunogenic proteins as shown in Fig. 5B. These results therefore suggest that native enolase is not immunogenic through natural contact and confirm the presence of native enolase in the saliva of males only.

4. Discussion

Owing to the importance of tick saliva in blood feeding, host immunity and pathogen transmission, our aim in this work was to identify the proteins that *O. moubata* secretes to this fluid and consequently inoculates into the host during feeding. To accomplish this goal, we carried out a proteomic study of the tick saliva instead of its SGE. In this way we could be certain that all the proteins identified were actually secreted and were not housekeeping/intracellular proteins released by the broken salivary gland cells, as may be the case of a fraction of the proteins present in the SGE.

Accordingly, pilocarpine- and dopamine-induced saliva was collected from tick mouthparts, carefully avoiding its contamination. The saliva from females and males was collected separately because prior observations had suggested that the protein composition of *O. moubata* saliva might differ between the sexes (unpublished data). Comparative analysis by SDS-PAGE of several pilocarpine- and dopamine-induced saliva batches demonstrated reproducibility between both protocols used to induce tick salivation and reproducibility among saliva batches from the same sex, confirming the difference in salivary composition between sexes (Fig. 1A).

Hence, we managed to collect up to 3 mg of saliva protein from each sex and equalized both samples in order to facilitate the identification of the low-abundance proteins. The need for saliva equalization was assumed on the basis of our previous experience with SGE, in which we observed that the hyperabundant TSGP1 lipocalin was hindering the detection of the poorly represented proteins [17-18]. As was the case for SGE, the TSGP1 lipocalin was the most abundant protein in the non-equalized saliva of females, and second to GAPDH in the non-equalized saliva of males. Accordingly, it was not surprising that when equalization had removed the “excess” of TSGP1 and other proteins from saliva the number of identified proteins increased significantly, particularly in females, where the ratio of new identifications (69.2%) was almost double that of males (38%) (Fig. 1B; Table 1).

The identification of 193 different proteins in the tick saliva was indicative of a broad and complex *O. moubata* saliva composition, in good agreement with the complexity of the argasid and ixodid sialomes described previously [2, 8, 9, 29, 30, 36]. However, when we looked at the identity of those proteins several issues arose that could be considered controversial.

First, we anticipated identifying the *O. moubata* salivary proteins previously described and uploaded in the databases, including some well known antihaemostatic and antiinflammatory agents such as ornithodorin, TAP and OMCI [37-39]. Thus, the failure to detect these proteins is difficult to explain. Although these proteins are assumed to be present in saliva at physiologically relevant concentrations, it may also be assumed that their identification in the native saliva samples might have been hindered by the overwhelming abundance of

proteins such as TSGP1. Regarding the equalized samples, since the equalization process requires large amounts of starting sample (10 mg according to the ProteoMiner manufacturer), working with 3 mg of saliva would had not favoured the identification of all the expected proteins. Furthermore, some proteins could have been lost along the sample lyophilisation and dialysis steps performed before equalization. Additionally, it could be also hypothesized that since saliva was collected at certain tick feeding time points, some of the known tick salivary proteins might not be present in a given saliva sample.

Second, among the proteins identified we obtained a very high ratio of housekeeping (77.5%) *versus* secreted (22.5%) proteins, in contrast to other soft-tick sialomes described, where secretory proteins represented more than 60% [29, 30, 36]. In our analysis, very few of the highly abundant secretory protein families in soft ticks were identified: i.e., no basic tail proteins, Kunitz-type protease inhibitors and metalloproteases; only those lipocalins for which sequences exist in the database. A possible reason for missing these proteins could be the high sequence diversity observed for many of the major secretory families in tick sialomes [2, 8, 9, 29, 30, 38] and the small number of *O. moubata* proteins and ESTs in the databases (128 and 95, respectively), which would have resulted in a lack of specific target sequences for comparative purposes. The 33% of unidentified protein hits in table 1 lend support to this idea. Hence, the low ratio of secreted *vs*. housekeeping proteins obtained here should be taken with caution.

In spite of the foregoing, it was not completely unexpected to find housekeeping/intracellular proteins in tick saliva [2, 40, 41]. It is increasingly recognized that some housekeeping/intracellular proteins can be secreted in unconventional ways to the extracellular milieu, where they play additional extracellular functions that are not always related to their intracellular functions [42]. Examples of such proteins in *O. moubata* saliva could be GAPDH and enolase. These proteins are well known cytoplasmic glycolytic enzymes but they have also been described as virulence-associated immunomodulatory and profibrinolytic agents in the extracellular milieu [43, 44]. Thus, the presence in tick saliva of proteins formerly designated as intracellular could help to identify novel secreted protein families and functions in tick sialomes. In accordance with this, the SecretomeP analysis predicted unconventional secretion mechanisms for one-third of these housekeeping/intracellular proteins, helping to explain their presence in saliva. However, SecretomeP did not predict any unconventional secretion mechanism for the other two-thirds of housekeeping/intracellular proteins. Among this second group of proteins we found a wide range of proteins known to be secreted in exosomes, including actin, enolase, fructose 1,6-bis phosphate aldolase, heat shock proteins 70 and 90, histones, pyruvate kinase, phosphoenolpyruvate carboxylase kinase, and ubiquitin C, among others (Tables 2 and 3). Exosomes are small membrane vesicles containing lipids, adhesion and intercellular signalling

molecules as well as RNAs, which eukaryotic cells secrete into their extracellular environment, entering body fluids. Exosome secretion is used more widely by cells and organisms than has previously been appreciated and is known to be involved in both physiological and pathological processes [45-49]. Thus, the finding in the saliva of *O. moubata* of a range of housekeeping/intracellular proteins known to be secreted by exosomes in other organisms suggests that this tick could also be using exosome secretion to produce saliva. Moreover, evidence for apocrine secretion of the cytoplasmic content in the salivary gland cells of argasid ticks has been reported [9], which would also contribute to explaining the presence of housekeeping/intracellular proteins in saliva, although this type of secretion would be very unspecific with respect which housekeeping proteins will actually be secreted.

Whereas the above reasons may explain the presence in the *O. moubata* saliva of a higher number of unconventionally than conventionally secreted proteins, the quantitative data drawn from the emPAI values and Sypro Ruby-stained gels indicate the opposite. That is, the relative abundance of conventionally secreted proteins was much higher than that of the housekeeping or unconventionally secreted ones, at least in female saliva (Figs. 3 and 4). These conventionally secreted proteins were mainly lipocalins such as TSGP1, TSGP4 and moubatin. Lipocalins are known to be abundantly expressed in tick saliva, playing important antihaemostatic and antiinflammatory functions at the tick bite site as scavengers of biogenic amines, leukotrienes and adenosine nucleotides. Accordingly, in light of previous reports it was not surprising to find them so abundantly expressed in the *O. moubata* saliva as well [17, 50-52]. Conversely, the low abundance of most of the housekeeping proteins identified in female saliva would suggest a non-targeted secretion (i.e., apocrine secretion), raising the question of whether such proteins would be of biological relevance at the tick feeding site or not. For instance, TSGP1, which possesses signal peptide, would represent more than 90% of the protein mass in female saliva while actin, without classical secretion signals, would not surpass the 0.6% (Fig. 4). In male saliva, however, the unusually secreted proteins were more abundant than the classically secreted ones, especially the group of enzymes involved in energy metabolism, such as GAPDH, FBA, enolase and PyK. In males, TSGP1 represents up to 31.5% of the protein mass, while GAPDH accounts for the 51.6% (Fig. 4). This suggests that these proteins would have been secreted to the saliva in a targeted way, and according to Ribeiro et al. [9] they would play some kind of antihaemostatic, anti-inflammatory or immunemodulatory role. Thus, future studies aimed at their functional characterization in this extracellular environment are needed. The production of recombinants and the identification of their vertebrate molecular targets may possibly be a useful starting point, as has been performed, for example, with the *S. bovis* enolase, which acts as a profibrinolytic plasminogen receptor when expressed on the tegument surface of this worm [53], and more recently with the *O. moubata* enolase, which also

act as a profibrinolytic plasminogen receptor, most likely helping the tick to maintain the fluidity of host blood during feeding [31].

The third controversial finding concerning the identity of the proteins found in the *O. moubata* saliva was the large difference observed between the protein composition of males and females, since only 5.2% of the proteins identified were shared by both sexes. The interpretation of this difference should be done cautiously because, as stated before, a range of the salivary proteins of *O. moubata* were not identified in the current study. Should these proteins be shared by both sexes, then the final difference between the sexes would be lower than that currently observed. However, this possibility does not rule out the difference observed in the current proteomic study, which is also supported by the different protein band patterns and protein identities shown in Figs. 1 and 4. Such a difference was unexpected for several reasons.

It is known that in hard ticks the anatomy and functions of their salivary glands differ between the sexes, male ticks having additional specific salivary gland acini. The feeding patterns between the sexes are also different since the males ingest very low or negligible amounts of blood as compared with the females [54]. In addition, several salivary gland genes are differentially expressed between males and females along the feeding process [55-57], and the effect of salivary gland extracts on the host immune response varies between males and females [58]. All of this can explain the observed differences in saliva composition between hard tick sexes. Noteworthy examples of these differences are the so-called Immunoglobulin-Binding Proteins (IGBPs), which are only secreted by males feeding adjacent to feeding females. These IGPBs are ingested by the females, helping them to prevent damage from host antibodies and hence enhancing their fecundity [35, 59].

As opposed to hard ticks, soft ticks are generally fast feeders and it is generally accepted that they have already the components of their saliva synthesized and ready to be secreted [54]. In addition, in soft ticks both sexes feed equally and *O. moubata* is no exception. *O. moubata* females and males ingest equivalent amounts of blood (corresponding to 1–3 times their body weight) and remain on the host for a similar time period to complete their blood meal (40–60 minutes) [16]. This means that both sexes are obliged to overcome the same defensive responses from the host to become engorged; consequently one would expect both sexes to secrete a similar array of anti-haemostatic salivary molecules. However, the results reported here suggest that both sexes secrete a different array of proteins and this raises the question of what the biological significance of this difference might be.

Also, although the classical histological and ultrastructural studies of argasid salivary glands (*Argas persicus*, *A. arboreus* and *O. moubata*) did not report differences between the sexes as regards their salivary gland structure [60-63], a more recent work by Mans et al. [64] revealed that the *O. savignyi* salivary glands seem to be more complex than previously thought, involving more granular cell types than those described on a morphological basis. These authors

suggest that alternative classification methodologies that rely on the physical expression patterns of the salivary gland proteome might be more reliable as markers of a specific granular cell type. Thus, it could be speculated that different granular cell types secreting different proteins in male and female *O. moubata* salivary glands would account for the difference between both saliva proteomes found here.

Regardless of the mechanism by which this differential secretion might be generated, the biological significance of such differences remains unknown. It could be related to the post-feeding processing of the ingested blood and/or to attraction and mating, which in *O. moubata* occurs soon after moulting, outside the host and before feeding [65]. However, for the time being neither our current results nor works cited in the literature offer evidence to support or rule out such a notion. Answering these questions will require more in-depth studies.

Finally, since the results of the present proteomic analysis of *O. moubata* saliva were somewhat controversial, we sought further confirmation of them. With this in mind, we analysed the presence/absence in the saliva samples of actin and enolase by western blot, using custom-made hyperimmune sera against the corresponding recombinant proteins. We chose actin and enolase as targets because: (i) they have been classified as intracellular/housekeeping, lacking prediction for unconventional secretion ways; (ii) while actin was found in both sexes, enolase was found in males only; and (iii) their whole cDNA coding sequences were available in GeneBank from other authors or from our own work [31]. The results of the immune-detection confirmed those of proteomics regarding actin and enolase and lent supplementary support to the main conclusions drawn from this work (see below). Furthermore, this experiment showed that not all the secreted proteins were immunogenic in natural contacts, confirming previous observations [19]. In the case of enolase and actin, this could be due to the fact that they are highly conserved proteins and perhaps the host does not recognize them as non-self. However, they were both recognized after forcing the immune system of the host by immunization with adjuvants. While interesting, this is not the first time this phenomenon has been observed with tick salivary proteins. Kotsyfakis et al. [66] reported a similar type of behaviour for the sialostatin L2 of *Ixodes scapularis*, introducing the concept of the "silent" salivary antigen and proposing these silent antigens as promising anti-tick vaccine targets. More recently, in *O. moubata* García-Varas et al. [16] found another "silent" salivary antigen, the so-called Om44, which is a P-selectin antagonist ligand that proved to be a good candidate target for anti-*O. moubata* vaccines.

4. Conclusions

Here we report a "first pass" proteome analysis of the saliva from a soft tick, *O. moubata*. We identified 193 different proteins showing that *O. moubata* saliva has a broad and

complex composition, in good agreement with the complexity of the argasid and ixodid sialomes described previously. Only 10 of these proteins appeared in both sexes, revealing a large difference in the saliva composition between males and females, which was further supported by differential immune detection. This is the first time this difference has been shown for a soft tick and needs further investigation to address its biological significance. Although the most abundant proteins in *O. moubata* saliva were some *bona fide* secreted proteins (mainly lipocalins), in terms of protein numbers the majority of the proteins identified were intracellular/housekeeping proteins. The presence of this kind of proteins in a secreted fluid indicated they were probably secreted by non-classical pathways and further suggested they might be playing unexpected functions at the tick-host interface. This will also require more investigation. These findings contribute to increasing our knowledge of soft tick sialomes and may help in the identification of novel secreted salivary proteins and functions at the tick-host interface.

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Figure captions

Figure 1. Silver stained 5-20% SDS-PAGE showing saliva from female (F) and male (M) *O. moubata* ticks. (A) Pilocarpine-induced saliva from four randomly selected batches of each sex. (B) Pooled saliva from female and male ticks before (native) and after equalization (equ); protein fraction that did not bind to the ProteoMiner® columns (unb).

Figure 2. Proteins found in female and male saliva were classified as possible secreted or putative housekeeping, and further in groups according to their function and/or protein family. Pie charts represent the percentage of proteins found in each group with respect to the total number of proteins in that category (in brackets).

Figure 3. Proteins identified in female and male saliva. The proteins are classified as secreted (gray background) or housekeeping (white background), and further in groups according to their function and/or protein family. Left chart: % protein number is the ratio between the numbers of proteins identified in each group with respect to the total number of proteins identified in each sex. Right chart: the emPAI percentage of each group is the sum of the % emPAI of each protein in that group. The % emPAI of each protein is calculated as the emPAI value of that protein divided by the sum of the emPAI values of all the proteins in each sex and multiplied by 100.

Figure 4. Sypro Ruby-stained one-dimensional gel electrophoresis of native female and male saliva. Left panel shows the molecular weight marker positions in the gel and their values in kDa. In the panels of females and males, the relative abundance of the Sypro Ruby-stained bands was calculated using the Image Lab software and annotated as percentage values on the left side of the lanes. The bands were excised according to the pattern depicted on the right side of the lanes and the proteins present in them were identified by LC-MS/MS and annotated to the corresponding band (Protein ID): α2MG, alpha-2-macroglobulin; ArK, arginine kinase; ENO, enolase; FBA, fructose 1,6-bisphophate aldolase; GAPDH, glyceraldehide 3-phosphate dehydrogenase; GlyPH, glycogen phosphorylase; HSP70, heat shock protein 70; LAP, lysosomal acid phosphatase; MOU, moubatin; PyK, pyruvate kinase; SSGP, secreted salivary gland peptide; TSGP1, tick salivary gland protein 1.

Figure 5. Western blot. Recombinant *O. moubata* actin (rActin) and native saliva samples from female (F) and male (M) *O. moubata* ticks were separated in 5-20% polyacrylamide gels, transferred to nitrocellulose sheets, and reacted against: (A) a rabbit anti-rActin polyclonal

846 serum; (B) a pool of two sera obtained from two pigs sensitized by natural infestations. The
847 molecular weight markers are included on the left side of the panel.
848
849 Figure 6. Western blot. Recombinant *O. moubata* enolase (rEno) and native saliva samples from
850 female (F) and male (M) *O. moubata* ticks were separated in 5-20% polyacrylamide gels,
851 transferred to nitrocellulose sheets and reacted against: (A) a rabbit anti-rEnolase (rEno)
852 polyclonal serum; (B) a pool of two sera obtained from two pigs sensitized by natural
853 infestations. The molecular weight markers are included on the left side of the panel

Table 1

Table 1. Number of protein hits and protein identifications in the saliva of *Ornithodoros moubata*.

		Female saliva		Male saliva		Both sexes
Data base (algoritm)		**Native**	**Equalized**	**Native**	**Equalized**	**Total**
EST_acari (Mascot)	Total protein hits	65	81	77	55	278
	Unidentified protein hits (%)	37 (57%)	26 (33%)	18 (23%)	10 (18%)	91 (33%)
	Identified proteins	28	55	59	45	187
NCBInr_metazoa (P. Pilot)	Identified proteins	17	52	45	40	154
EST-acari + NCBInr_metazoa	Total identified proteins	45	107	104	85	341
	Non-redundant in each sample	36	82	51	34	203
	Non-redundant by each sex	118		85		-
	Total non-redundant	-		-		193

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Table 2. Proteins identified in native and equalized saliva from female *Ornithodoros moubata* ticks by LC-MS/MS. Proteins were classified as either secretory or housekeeping and further in groups based on function and/or protein families. Numbers in brackets indicate the number of non-redundant identifications inside each category/group. Inside groups, proteins are in ordered in decreasing scoring. Scores without decimals correspond to MASCOT-derived identifications; scores with decimals belong to Protein Pilot-derived identifications. PN, number of unique matched peptides. emPAI, exponentially modified protein abundance index. % emPAI, (percentage of exponentially modified protein abundance index) was calculated by dividing the emPAI value of a protein by the sum of all emPAI values multiplied by 100. MW, molecular weight (kDa) as predicted by the Uniprot web server (www.uniprot.org). Signal P, presence of classical secretion signal. SecretomeP, prediction of non-classical protein secretion. TMH, number of predicted transmembrane helices. GPI^n, potential site for GPI-anchor. Underlined names indicate proteins that were identified only in equalized saliva; proteins marked with asterisk (*) were identified in both sexes.

PROTEIN NAME	DATABASE ID NO.	SPECIES	SCORE	PN	emPAI	% emPAI	MW	Signal P	Secretome P	TMH/ GPI	Match to 1D-PAGE LC-MS/MS (Fig. 4)
PUTATIVE SECRETED PROTEINS (25)											
Serine proteases (3)											
factor D-like protein (serine proteinase)	AAO12856.1	*Dermacentor andersoni*	89	3	-	-	40.9	Yes			
serine proteinase, putative	XP_002409528.1	*Ixodes scapularis*	48	2	-	-	26.4			1 TMH	
urokinase-type plasminogen activator	XP_001848040.1	*Culex quinquefasciatus*	1.4	1	-	-	28.1	Yes	Yes		
Lipocalins (3)											
TSGP1 *	ADK94457.1	*Ornithodoros moubata*	537	79	3.66	89.92	21.7	Yes	Yes		yes
TSGP4	AAN76831.1	*Ornithodoros savignyi*	52	3	0.18	4.42	19.3	Yes	Yes		
moubatin *	AAA29432.1	*Ornithodoros moubata*	13.66	13	0.23	5.65	18.8	Yes	Yes		yes
Protease inhibitors (2)											
thyropin precursor (cistatin)	AAS01022.1	*Ornithodoros moubata*	172	12	-	-	7.9	Yes		1 TMH	yes
collagen alpha-3(VI) chain precursor (contains 1BPTI/ Kunitz inhibitor domain)	NP_990865.1	*Gallus gallus*	1.35	1	-	-	13.9	Yes			
Glycine rich superfamily (2)											
glycine rich secreted salivary gland protein	AAT75325.1	*Ixodes scapularis*	60	2	-	-	31.7	Yes	Yes		
cement protein	DAA34732.1	*Amblyomma variegatum*	51	3	-	-	39.9	Yes	Yes		

Mucins (2)											
proteophosphoglycan, putative	XP_002399267.1	*Ixodes scapularis*	56	3	-	-	21.9		Yes		
mucin-7, putative	XP_002406133.1	*Ixodes scapularis*	48	3	-	-	23.6		Yes		
Nucleotidases (2)											
5'-nucleotidase/apyrase	ABS30897.1	*Ornithodoros savignyi*	4.33	2	-	-	65.1	Yes			yes
5'-nucleotidase III (subfamily IE hydrolase)	XP_311397.4	*Anopheles gambiae*	2	1	-	-	34.4				
Immunity related (2)											
alpha-2-macroglobulin precursor *	AAN10129.1	*Ornithodoros moubata*	50.37	21	-	-	165.1	Yes	Yes	1 TMH	yes
lectin 1 putative immunolectin (C-type lectin)	AAV91436.1	*Lonomia obliqua*	2	1	-	-	60.3				
Phosphatases (1)											
lysosomal acid phosphatase *	XP_002410320.1	*Ixodes scapularis*	126	6	-	-	42.3	Yes	Yes		
Secreted conserved proteins (unknown function) (8)											
hypothetical protein	XP_002399190.1	*Ixodes scapularis*	67	7	-	-	13.9	Yes			
secreted PAPA repeat protein	DAA34610.1	*Amblyomma variegatum*	65	3	-	-	25.8	Yes	Yes	1 TMH	
hypothetical secreted protein 108	DAA34554.1	*Amblyomma variegatum*	64	2	-	-	15.2	Yes	Yes	GPI[121]	
secreted salivary gland peptide	XP_002406260.1	*Ixodes scapularis*	61	2	-	-	25.0				
secreted protein, putative	XP_002408924.1	*Ixodes scapularis*	58	2	-	-	7.5			2 TMH	
secreted protein, putative	XP_002399632.1	*Ixodes scapularis*	55	3	-	-	20.6	Yes	Yes		
secreted protein, putative	XP_002402709.1	*Ixodes scapularis*	52	2	-	-	38.3		Yes		
F-box/LRR-repeat protein, putative	XP_002403227.1	*Ixodes scapularis*	48	2	-	-	45.3		Yes		
POSSIBLE HOUSEKEEPING PROTEINS (92)											
Signal transduction (14)											
guanylate-binding protein	XP_002408177.1	*Ixodes scapularis*	61	2	-	-	60.67			2 TMH	
cyclin-dependent kinase-2 interacting protein	XP_002399373.1	*Ixodes scapularis*	59	6	-	-	24.07				
toll, putative	XP_002406802.1	*Ixodes scapularis*	58	7	-	-	41.69			1 TMH	
GDP-mannose 4,6-dehydratase	NP_001134845.1	*Salmo salar*	56	2	-	-	47.65				
hypothetical protein RhoGEF domain	XP_001649435.1	*Aedes aegypti*	50	3	-	-	205.78		Yes		
phosphoenolpyruvate carboxylase kinase *	XP_002413374.1	*Ixodes scapularis*	56	2	-	-	61.04				

calumenin, putative	XP_002407941.1	*Ixodes scapularis*	49	2	-	-	40.12	Yes		
leucine rich repeat C-terminal domain	XP_002133864.1	*Drosophila pseudoobscura*	2.31	1	-	-	202.84	Yes	Yes	9 TMH
guanylate cyclase	XP_001663219.1	*Aedes aegypti*	2	1	-	-	70.17			
mitogen-activated protein kinase 8	NP_571796.1	*Danio rerio*	2	1	-	-	44.14			
A-kinase anchor protein	AAD39150.1	*Rattus norvegicus*	1.7	1	-	-	254.35		Yes	
GTPase-activating protein, putative.	XP_002426989.1	*Pediculus humanus*	1.55	1	-	-	178.86		Yes	
7-transmembrane G-protein-coupled receptor *	XP_002632572.1	*Caenorhabditis briggsae*	1.39	1	-	-	92.86			7 TMH
amyloid beta A4 precursor protein-binding family A	XP_002914782.1	*Ailuropoda melanoleuca*	1.31	1	-	-	37.51		Yes	
Nuclear regulation (13)										
DNA double-strand break repair rad50 ATPase	XP_002407401.1	*Ixodes scapularis*	63	3	-	-	59.64			
zinc finger CCHC domain-containing protein 4-like	XP_003411427.1	*Loxodonta africana*	61	4	-	-	59.46			
apoptosis-promoting RNA-binding protein TIA-1	XP_002408889.1	*Ixodes scapularis*	55	5	-	-	35.47		Yes	
core histone H2A/H2B/H3/H4	XP_002399568.1	*Ixodes scapularis*	52	2	-	-	15.33			
DNA repair protein RAD51/RHP55, putative	XP_002405184.1	*Ixodes scapularis*	52	6	-	-	38.31			
hypothetical protein	XP_002108586.1	*Trichoplax adhaerens*	52	2	-	-	119.74			
cell division cycle 2-like	AAY66874.1	*Ixodes scapularis*	50	2	-	-	11.33		Yes	
RNA-binding protein musashi *	XP_002412054.1	*Ixodes scapularis*	48	2	-	-	28.82		Yes	
histone	XP_002413715.1	*Ixodes scapularis*	48	3	-	-	24.86		Yes	
recombination activating protein 1 (RAG1)	ABG47733.1	*Glyptothorax trilineatus*	2,15	1	-	-	43.97			
alphaA-crystallin-binding protein 1	AAA98810.1	*Mus musculus*	2	1	-	-	288.34		Yes	
TRAP-like (trr-1) kinase	NP_001022032.1	*Caenorhabditis elegans*	1.73	1	-	-	465.55			
suppressor of sable	XP_002099698.1	*Drosophila yakuba*	1.43	3	-	-	147.23		Yes	
Protein synthesis, modification and export machinery (10)										
glycosyltransferase	XP_002405751.1	*Ixodes scapularis*	53	4	-	-	40.08			
ATP-dependent RNA helicase DDX56	NP_001231472.1	*Sus scrofa*	53	2	-	-	61.32			
ribophorin	XP_002409944.1	*Ixodes scapularis*	51	6	-	-	68.44	Yes		1 TMH
chaperonin containing TCP1, subunit 3 (gamma)	XP_003415199.1	*Loxodonta africana*	50	2	-	-	60.59			
serine/threonine kinase NLK	XP_001662367.1	*Aedes aegypti*	50	2	-	-	36.28		Yes	

cyclophilin B precursor	XP_002410624.1	*Ixodes scapularis*	48	3	-	-	21.47	Yes			
ADP ribosylation factor 79F *	ABI52727.1	*Argas monolakensis*	48	2	-	-	20.58				
SH2 domain binding protein (TPR superfamily).	ACY44038.1	*Abacion magnum*	1.7	1	-	-	19.37				
probable rRNA-processing protein EBP2-like	BAH71299.1	*Acyrthosiphon pisum*	1.6	1	-	-	33.39		Yes		
Rab5 GDP/GTP exchange factor	NP_055319.1	*Homo sapiens*	1.5	1	-	-	79.37				
Transporters (10)											
vitellogenin *	BAH02666.2	*Ornithodoros moubata*	735	45	-	-	205.06	Yes	Yes		
hemelipoglycoprotein precursor	XP_002411431.1	*Ixodes scapularis*	65	5	-	-	56.24				
hemelipoglycoprotein precursor	XP_002411435.1	*Ixodes scapularis*	58	2	-	-	177.66	Yes	Yes		
innexin inx3-like	XP_003427733.1	*Nasonia vitripennis*	52	3	-	-	44.76			4 TMH	
nuclear pore complex protein nup98	XP_002415833.1	*Ixodes scapularis*	49	5	-	-	146.05		Yes		
ATP-binding cassette sub-family A member 3-like	XP_001947916.2	*Acyrthosiphon pisum*	48	5	-	-	197.53			14 TMH	
Sec14p-like lipid-binding domain containing protein	XP_002404368.1	*Ixodes scapularis*	48	2	-	-	80.58				
Bestrophin-4	ABG02138.2	*Drosophila melanogaster*	1.57	1	-	-	64.06			5 TMH	
2-oxoglutarate/malate carrier protein-like	XP_002610854.1	*Branchiostoma floridae*	1.32	1	-	-	34.32			1 TMH	
protein canopy homolog 1 precursor	NP_001088889.1	*Xenopus laevis*	1.32	1	-	-	20.71	Yes			
Cytoskeletal proteins (5)											
actin *	BAE46505.1	*Ornithodoros moubata*	87	6	-	-	41.84				yes
paramyosin, putative	XP_002407400.1	*Ixodes scapularis*	63	3	-	-	56.83				
microtubule-actin cross-linking factor 1-like	XP_003454660.1	*Oreochromis niloticus*	60	2	-	-	855.23				
septin-1-like	XP_003493686.1	*Bombus impatiens*	59	3	-	-	41.09				
dynein heavy chain, isoform C	NP_001163759.1	*Drosophila melanogaster*	2.4	1	-	-	580.46				yes
Detoxification (5)											
alcohol dehydrogenase, short chain	XP_002406421.1	*Ixodes scapularis*	71	5	-	-	25.76				
cytochrome P450	XP_002434947.1	*Ixodes scapularis*	61	2	-	-	60.22			2 TMH	
monooxygenase	XP_002412032.1	*Ixodes scapularis*	61	3	-	-	65.82		Yes		
sulfotransferase, putative	XP_002412156.1	*Ixodes scapularis*	49	2	-	-	31.23				
short-chain dehydrogenase	EAT45862.1	*Aedes aegypti*	2	1	-	-	37.61				

Proteasome machinery (5)										
goliath E3 ubiquitin ligase-like	XP_966546.1	*Tribolium castaneum*	56	3	-	-	52.08	Yes	Yes	1 TMH
ubiquitin protein ligase	XP_002414996.1	*Ixodes scapularis*	51	5	-	-	19.72		Yes	
ubiquitin carboxyl-terminal hydrolase	XP_002636074.1	*Caenorhabditis briggsae*	2	1	-	-	46.20		Yes	
F-box and WD domain protein	XP_002406691.1	*Ixodes scapularis*	1.6	1	-	-	58.09			
E3 ubiquitin-protein ligase hyd	NP_524296.2	*Drosophila melanogaster*	1.43	1	-	-	318.88		Yes	
Transcription machinery (4)										
conserved hypothetical protein	XP_002407059.1	*Ixodes scapularis*	61	4	-	-	28.36			
PR domain zinc finger protein 2-like	XP_002925632.1	*Ailuropoda melanoleuca*	1.91	1	-	-	186.30			
Elav-like family member 6-like	XP_002919757.1	*Ailuropoda melanoleuca*	1.7	1	-	-	49.89			
chromodomain helicase-DNA-binding protein 3	XP_001846200.1	*Culex quinquefasciatus*	1.52	1	-	-	223.88			
Metabolism, energy (4)										
electron transfer flavoprotein subunit alpha	XP_624102.1	*Apis mellifera*	57	3	-	-	35.38			
cytochrome C oxidase subunit Va	XP_002409979.1	*Ixodes scapularis*	55	2	-	-	17.46			
phosphorylase kinase gamma subunit	XP_002400416.1	*Ixodes scapularis*	54	3	-	-	44.78			
acyl-CoA synthetase	XP_002408024.1	*Ixodes scapularis*	50	3	-	-	47.34			
Metabolism, nucleotide and carbohydrate (3)										
glucosamine-fructose-6-P- aminotransferase	XP_765503.1	*Theileria parva*	49	2	-	-	90.38			
adenine phosphoribosyltransferase isoform b	NP_001025189.1	*Homo sapiens*	1.87	1	-	-	14.55			
Adenosine/AMP deaminase	AAD52851.1	*Glossina morsitans*	1.4	1	-	-	58.22	Yes		
Extracellular matrix and adhesion (2)										
ankyrin repeat domain-containing protein	XP_003486678.1	*Bombus impatiens*	48	4	-	-	23,736			
precollagen-NG	AAM34599.1	*Mytilus galloprovincialis*	2.03	1	-	-	79,251	Yes	Yes	
Unknown conserved (17)										
hypothetical protein	XP_002409579.1	*Ixodes scapularis*	66	3	-	-	21.07		Yes	
conserved hypothetical protein	XP_002429027.1	*Pediculus humanus*	60	2	-	-	134.22		Yes	
conserved hypothetical protein	XP_002399363.1	*Ixodes scapularis*	57	3	-	-	21.58		Yes	
conserved hypothetical protein	XP_002411579.1	*Ixodes scapularis*	57	3	-	-	66.55			

hypothetical protein	XP_002411365.1	*Ixodes scapularis*	57	2	-	-	13.18			2 TMH	
hypothetical protein	XP_002402844.1	*Ixodes scapularis*	57	2	-	-	24.48			4 TMH	
hypothetical protein	XP_002412482.1	*Ixodes scapularis*	54	3	-	-	23.48				
hypothetical protein	XP_002401289.1	*Ixodes scapularis*	52	2	-	-	10.88				
hypothetical protein	XP_002433579.1	*Ixodes scapularis*	52	2	-	-	12.68				
hypothetical protein	XP_002408728.1	*Ixodes scapularis*	51	4	-	-	6.24		Yes		
RING finger motif (Zinc finger) containing protein	XP_002405290.1	*Ixodes scapularis*	51	2	-	-	16.16				
hypothetical protein	XP_002433647.1	*Ixodes scapularis*	50	3	-	-	12.82				
conserved hypothetical protein	XP_002406269.1	*Ixodes scapularis*	48	2	-	-	10.40				
uncharacterized protein	XP_002100206.1	*Drosophila yakuba*	2.25	1	-	-	89.55	Yes	Yes		
retrotransposon-like 1	NP_001127830.4	*Sus scrofa*	2.04	1	-	-	153.28			1 TMH	
acetylcholinesterase, putative	XP_002409706.1	*Ixodes scapularis*	1.7	1	-	-	51.14				
uncharacterized	XP_002095878.1	*Drosophila yakuba*	1.31	1	-	-	29.53		Yes		
HOST ORIGIN PROTEINS (1)											
serum albumin	NP_001075813.1	*Oryctolagus cuniculus*	9.47	4	-	-	68.9	Yes			Yes

Table 3.docx
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Table 3. Proteins identified in native and equalized saliva from male *Ornithodoros moubata* ticks by LC-MS/MS. Proteins were classified as either secretory or housekeeping and further in groups based on function and/or protein families. Numbers in brackets indicate the number of non-redundant identifications inside each category/group. Inside groups, proteins are in ordered in decreasing scoring. Scores without decimals correspond to MASCOT-derived identifications; scores with decimals belong to Protein Pilot-derived identifications. PN, number of unique matched peptides. emPAI, exponentially modified protein abundance index. % emPAI, (percentage of exponentially modified protein abundance index) was calculated by dividing the emPAI value of a protein by the sum of all emPAI values multiplied by 100. MW, molecular weight (kDa) as predicted by the Uniprot web server (www.uniprot.org). Signal P, presence of classical secretion signal. SecretomeP, prediction of non-classical protein secretion. TMH, number of predicted transmembrane helices. GPI^{n}, potential site for GPI-anchor. Underlined names indicate proteins that were identified only in equalized saliva; proteins marked with asterisk (*) were identified in both sexes.

PROTEIN NAME	DATABASE ID NO.	SPECIES	SCORE	PN	emPAI	% emPAI	MW	Signal P	Secretome P	TMH/ GPI	Match to 1D-PAGE LC-MS/MS (Fig. 4)
PUTATIVE SECRETED PROTEINS (20)											
Serine proteases (2)											
trypsin-like serine protease	XP_002413603.1	*Ixodes scapularis*	51	3	-	-	26.58	Yes			
cationic trypsinogen precursor	AAA30900.1	*Canis sp.*	1.92	1	-	-	26.17	Yes	Yes		
Other peptidases (2)											
similar to ADAM metallopeptidase	XP_002168387.1	*Hydra magnipapillata*	2	1	-	-	58.10		Yes	2 TMH	
angiotensin-converting enzyme	XP_001659916.1	*Aedes aegypti*	2	1	-	-	70.14				
Lipocalins (2)											
TSGP1 *	ADK94457.1	*Ornithodoros moubata*	349	43	2.17	29.60	21.71	Yes	Yes		yes
moubatin *	AAA29432.1	*Ornithodoros moubata*	9.65	10	0.1	1.36	18.82	Yes	Yes		yes
Immunity related (2)											
alpha-2-macroglobulin precursor *	AAN10129.1	*Ornithodoros moubata*	61	2	-	-	165.07	Yes	Yes	1 TMH	yes
hemolin, putative	XP_002407721.1	*Ixodes scapularis*	50	4	-	-	29.79		Yes		
Glycine rich superfamily (1)											
structural constituent of cuticle, putative	XP_002410255.1	*Ixodes scapularis*	69	2	-	-	51.18	Yes	Yes		
Mucins (1)											

mucin/peritrophin-like protein precursor	AAS01023.1	*Ornithodoros moubata*	2	1	-	-	19.5	Yes	Yes		yes
Protease inhibitors (1)											
similar to serine proteinase inhibitor	XP_002199473.1	*Taeniopygia guttata*	1.35	1	-	-	43.44				
Hydrolases (1)											
epoxide hydrolase 1	XP_001489035.1	*Equus caballus*	54	2	-	-	52.51	Yes			
Phosphatases (1)											
lysosomal acid phosphatase *	XP_002410320.1	*Ixodes scapularis*	65	3	0.1	1.36	42.33	Yes	Yes		yes
Secreted conserved proteins (unknown function) (7)											
unknown secreted protein DS-1	AAS94229.1	*Ornithodoros moubata*	137	3	-	-	38.8	Yes			
unknown secreted protein PK-26	AAS94230.1	*Ornithodoros moubata*	137	3	-	-	39.0	Yes			
conserved hypothetical protein	XP_002404867.1	*Ixodes scapularis*	91	2	0.11	1.50	18.83	Yes			
fasciclin domain-containing protein, putative	XP_002399918.1	*Ixodes scapularis*	59	2	-	-	37.56	Yes	Yes		
hypothetical protein	XP_002402150.1	*Ixodes scapularis*	58	3	-	-	52.04	Yes		5 TMH	
secreted salivary gland peptide, putative	XP_002400957.1	*Ixodes scapularis*	54	2	-	-	16.16	Yes	Yes	6 TMH	
salivary gland peptide, putative	XP_002412511.1	*Ixodes scapularis*	50	2	0.08	1.09	28.94	Yes	Yes	4 TMH	yes
POSSIBLE HOUSEKEEPING PROTEINS (63)											
Protein synthesis, modification and export machinery (10)											
arginine kinase	NM_001104086.1	*Drosophila melanogaster*	213	5	0.29	3.95	39.9 - 61.2				yes
HSP70 family member	BK007244.1	*Amblyomma variegatum*	69	3	-	-	56.19				
protein folding, similar to HSP90	XM_002413104.1	*Ixodes scapularis*	62	4	-	-	90.28				
methionyl-tRNA synthetase, putative	XM_002407084.1	*Ixodes scapularis*	60	3	-	-	74.24				
ADP ribosylation factor 79F *	DQ886810.1	*Argas monolakensis*	60	2	-	-	20.58				
thioredoxin H2 protein, putative	XM_002401871.1	*Ixodes scapularis*	54	2	-	-	24.14	Yes			yes
endophilin A, putative	XM_002423258.1	*Pediculus humanus*	53	2	-	-	40.17		Yes		
3D7 sec-1 family protein	XM_001352180.1	*Plasmodium falciparum*	51	2	-	-	135.98		Yes		
40S ribosomal protein SA	EF633966.1	*Ornithodoros parkeri*	48	2	-	-	33.07		Yes		
microtubule affinity-regulating kinase 1	BC072186.1	*Xenopus laevis*	2	1	-	-	88.62		Yes		
Metabolism, lipid, nucleotide and carbohydrate (8)											

glycogen phosphorylase, putative	XP_002408095.1	*Ixodes scapularis*	77	2	*0.11*	1.50	94.28				yes
triosephosphate isomerase	XP_002411305.1	*Ixodes scapularis*	57	3	-	-	26.93				
ptm1, putative	XP_002436068.1	*Ixodes scapularis*	55	5	-	-	52,399	Yes		5 TMH	
imidazolone-5-propionate hydrolase	XP_002413459.1	*Ixodes scapularis*	54	3	-	-	18.12				
glycine/serine hydroxymethyltransferase	XP_002400747.1	*Ixodes scapularis*	49	4	-	-	51.69				
fatty acyl-CoA elongase, putative	XP_002434209.1	*Ixodes scapularis*	49	2	-	-	31.81			5 TMH	
triosephosphate isomerase	XP_002166707.1	*Bombyx mori*	2.02	1	-	-	26.78		Yes		
arylformamidase (esterase-lipase)	EEC09597.1	*Ixodes scapularis*	1.53	1	-	-	31.95				
Metabolism, energy (6)											
GAPDH	ACH88101.1	*Dermacentor variabilis*	269	15	1,46	19.91	35.95		Yes		yes
fructose 1,6-bisphosphate aldolase	XP_002411768.1	*Ixodes scapularis*	255	5	1,71	23.33	39.44		Yes		yes
enolase	ADD91327.1	*Ornithodoros moubata*	250	5	0,56	7.64	46.96				yes
glyoxylate/hydroxypyruvate reductase	XP_002407150.1	*Ixodes scapularis*	159	4	0,26	3.54	35.92				
pyruvate kinase	XP_002407464.1	*Ixodes scapularis*	101	7	0,13	1.77	58.48				yes
NADH-ubiquinone oxidoreductase 24 kDa	XP_002424632.1	*Pediculus humanus*	54	2	-	-	27.25				
Signal transduction (5)											
seven transmembrane receptor	XP_002436127.1	*Ixodes scapularis*	60	4	-	-	48.46			6 TMH	
phosphoenolpyruvate carboxylase kinase *	XP_002413374.1	*Ixodes scapularis*	60	4	-	-	61.04				
RAS family protein, putative	XP_002433782.1	*Ixodes scapularis*	55	2	-	-	20.64				
agouti-related protein	AAG09464.1	*Sus scrofa*	2	1	-	-	13.20				
7-transmembrane G-protein-coupled receptor *	XP_002632572.1	*Caenorhabditis briggsae*	1.62	1	-	-	37.50			7 TMH	
Cytoskeletal proteins (3)											
beta-actin	ABP01547.2	*Dermacentor variabilis*	87	3	0.14	1,91	41.82				yes
actin *	BAE46505.1	*Ornithodoros moubata*	3.89	2	-	-	41.84				yes
formin 1	NP_989754.1	*Gallus gallus*	1.43	1	-	-	135.24		Yes		
Nuclear regulation (3)											
RNA-binding protein musashi *	XP_002412054.1	*Ixodes scapularis*	56	3	-	-	28,815		Yes		
ATPase involved in DNA replication and repair	XP_002166707.1	*Hydra magnipapillata*	2	1	-	-	109,034		Yes		

DNA polymerase accessory subunit	AAC47536.1	*Drosophila melanogaster*	1.4	1	-	-	41,05			
Proteasome machinery (3)										
ubiquitin C	NP_066289.2	*Homo sapiens*	68	3	-	-	77.04			
polyubiquitin (WubiG)	ABU40645.1	*Triticum aestivum*	58	2	-	-	42.55			
COP9 signalosome complex subunit	XP_002423731.1	*Pediculus humanus corporis*	50	2	-	-	36.13			
Transporters (3)										
vitellogenin *	BAH02666.2	*Ornithodoros moubata*	267	9	-	-	205.06	Yes	Yes	
ferritin	AAC19132.1	*Ornithodoros moubata*	2	1	-	-	19.96			
Sugar phosphate exchanger 2	XP_003493559.1	*Bombus impatiens*	1.4	1	-	-	56.75			12 TMH
Transcription (2)					-	-				
Insulin protein enhancer protein	XP_002575133.1	*Schistosoma mansoni*	65	2	-	-	43.83		Yes	
isoform CRA_b (DNA integrase)	EAW82317.1	*Homo sapiens*	1.4	1	-	-	61.82			
Detoxification (1)										
nicotinate phosphoribosyltransferase	XP_623279.1	*Apis mellifera*	50	2	-	-	62.17			
Extracellular matrix and adhesion (1)										
opioid-binding protein/cell adhesion molecule	XP_003398771.1	*Bombus terrestris*	1.7	1	-	-	36.31	Yes		GPI299
Nuclear export machinery (1)										
nucleoporin Nup37, putative	XP_002404500.1	*Ixodes scapularis*	60	3	-	-	35.72		Yes	
Unknown conserved (17)										
zinc finger protein	NP_001037823.1	*Ciona intestinalis*	66	3	-	-	146.32		Yes	
WD repeat domain 33 (WDR33)	XP_001504985.1	*Equus caballus*	61	2	-	-	145.15		Yes	
conserved hypothetical protein	XP_002408103.1	*Ixodes scapularis*	60	3	-	-	56.93		Yes	
coiled-coil domain containing 55	XP_868363.2	*Canis lupus familiaris*	58	6	-	-	66.04		Yes	
hypothetical protein	XP_002416462.1	*Ixodes scapularis*	55	3	-	-	8.80			
conserved hypothetical protein	XP_002407001.1	*Ixodes scapularis*	55	3	-	-	24.12		Yes	
hypothetical protein	XP_002415025.1	*Ixodes scapularis*	54	3	-	-	16.23			1 TMH
hypothetical protein	XP_002434131.1	*Ixodes scapularis*	53	3	-	-	13.04		Yes	

conserved hypothetical protein	XP_002409499.1	*Ixodes scapularis*	53	3	-	-	33.09				
hypothetical protein	XP_002405948.1	*Ixodes scapularis*	52	3	-	-	7.39				
conserved hypothetical protein	XP_002406615.1	*Ixodes scapularis*	51	2	-	-	91.12				
conserved hypothetical protein	XP_002404459.1	*Ixodes scapularis*	51	2	-	-	34.84				
membrane protein, putative	XP_002408075.1	*Ixodes scapularis*	50	6	-	-	26.35	Yes		2 TMH	
hypothetical protein	XP_002403222.1	*Ixodes scapularis*	50	3	0,11	1.50	11.60			2 TMH	
conserved hypothetical protein	XP_001862194.1	*Culex quinquefasciatus*	1.75	1	-	-	128.08		Yes		
hypothetical protein AaeL_AAEL013784	XP_001657022.1	*Aedes aegypti*	1.72	1	-	-	66.21			1 TMH	
RWD domain-containing protein 1	ACQ58081.1	*Anoplopoma fimbria*	1.71	1	-	-	27.84				
HOST ORIGIN PROTEINS (2)											
alpha-hemoglobin	B8K132	*Oryctolagus cuniculus*	9.12	5	-	-	15.61				yes
beta-hemoglobin	K03256.1	*Oryctolagus cuniculus*	6.72	3	-	-	16.13				yes

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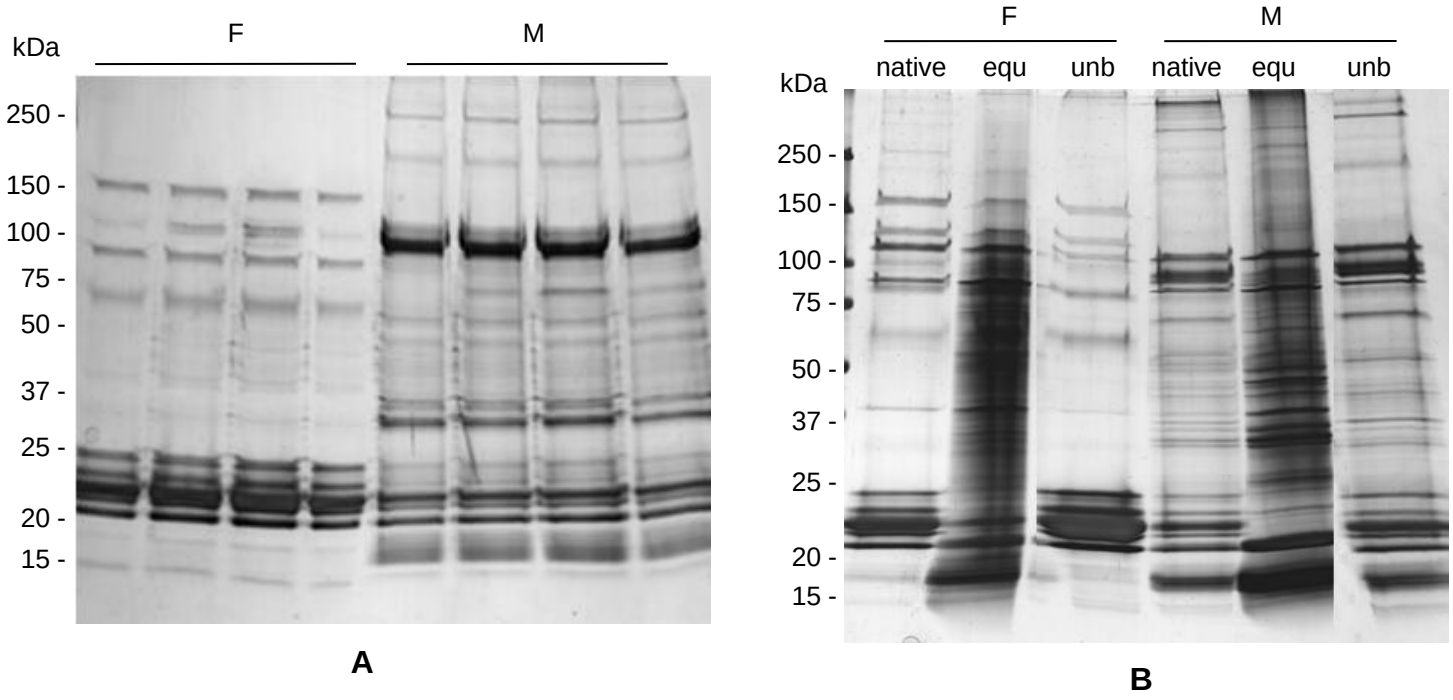
Figure 1



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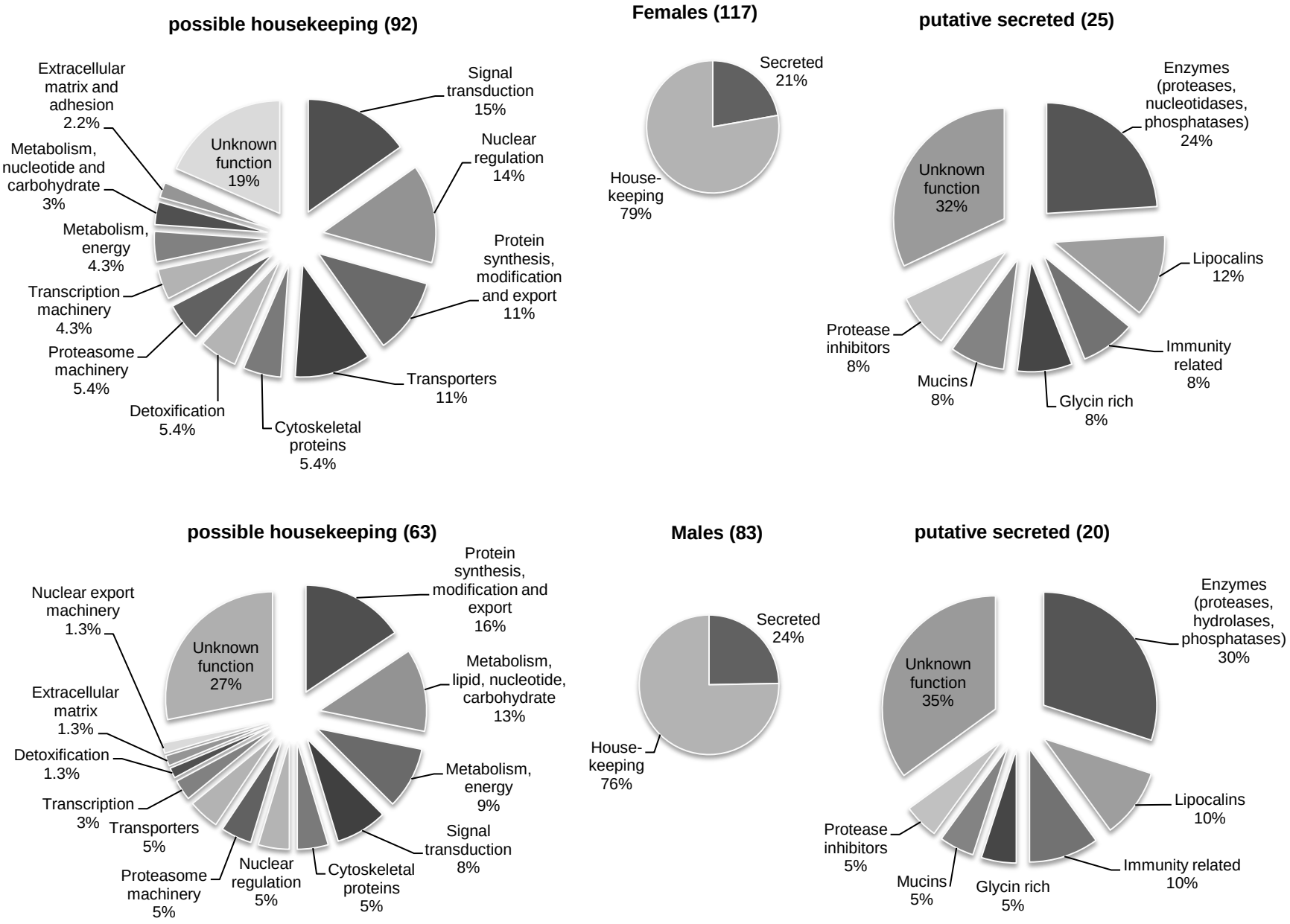

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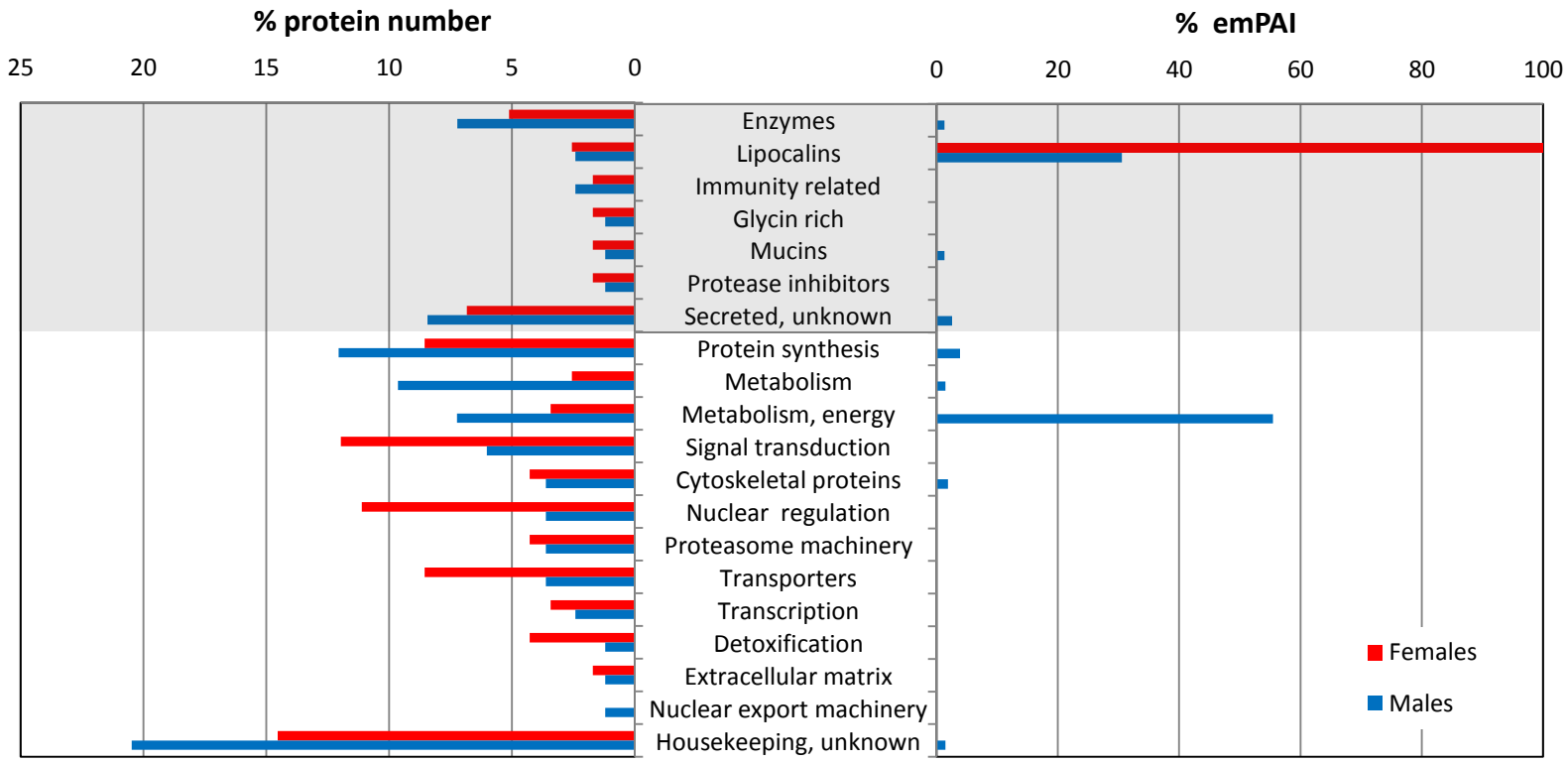

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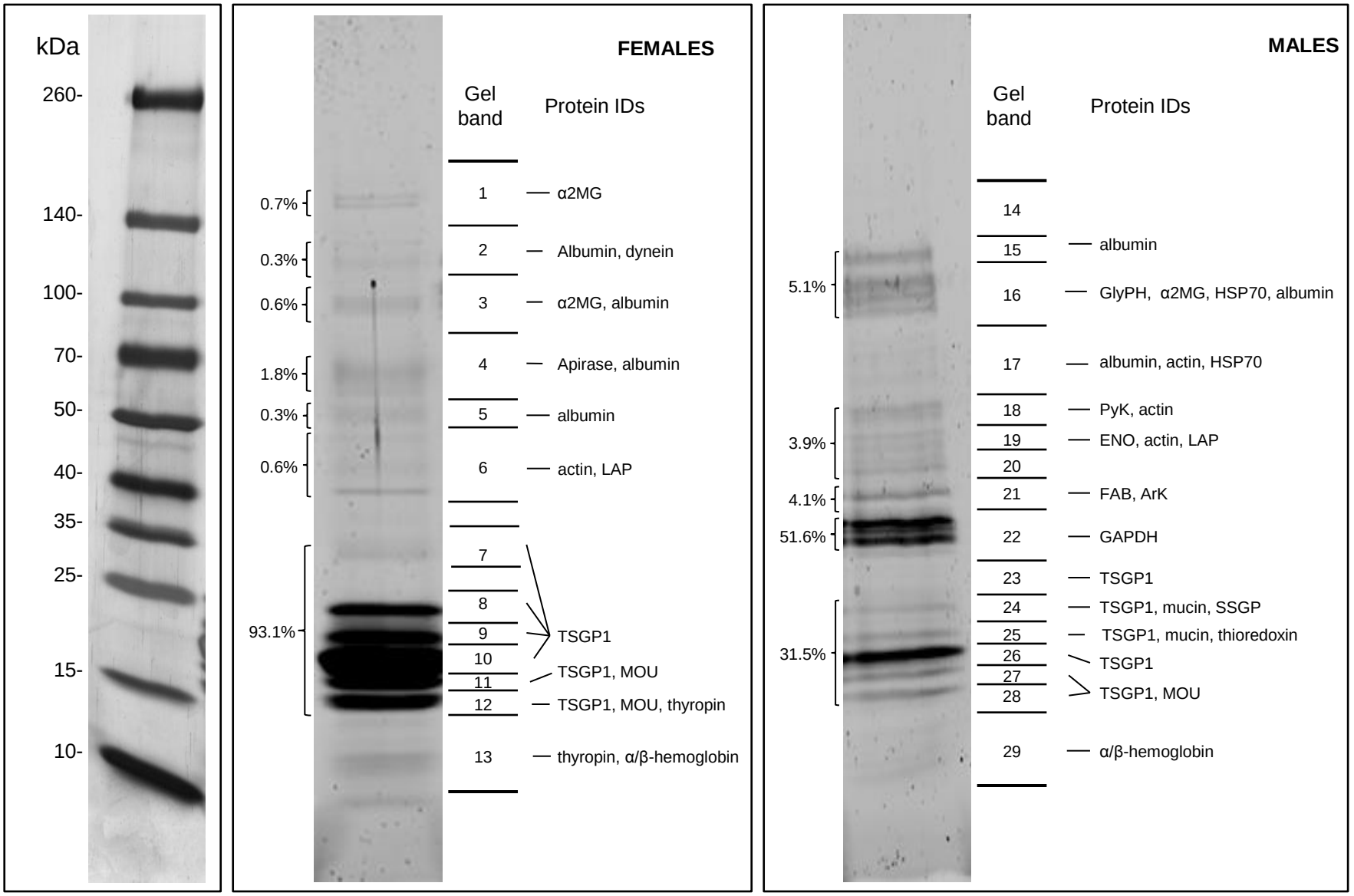

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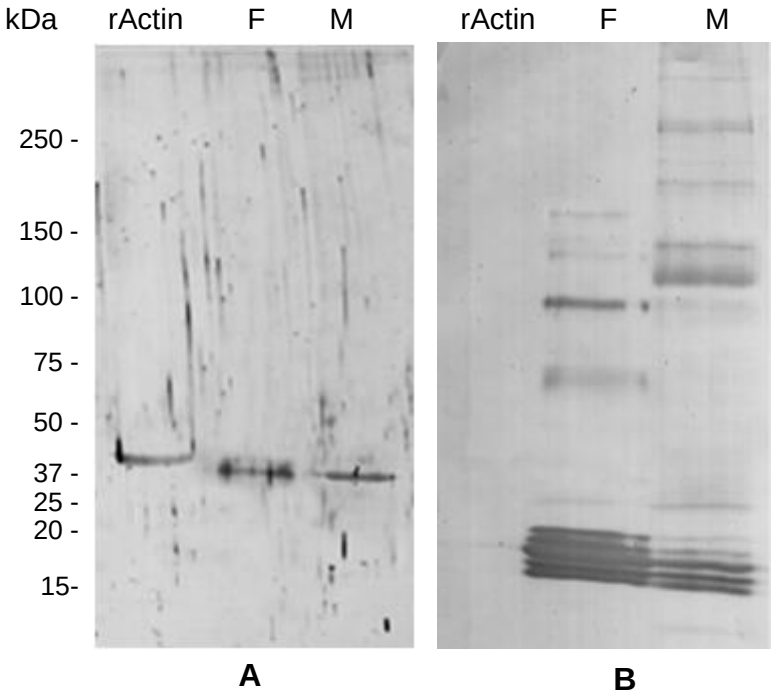

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